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Forced Retroevolution of an RNA Bacteriophage

Normunds Licis,* Zita Balklava,* and Jan Van Duint[1]

*Biomedical Research and Study Centre, University of Latvia, Ratsupites 1, LV 1067, Riga, Latvia; and †Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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The operator hairpin ahead of the replicase gene in RNA bacteriophage MS2 contains overlapping signals for binding the coat protein and ribosomes. Coat protein binding inhibits further translation of the gene and forms the first step in capsid formation. The hairpin sequence was partially randomized to assess the importance of this structure element for the bacteriophage and to monitor alternative solutions that would evolve on the passaging of mutant phages. The evolutionary reconstruction of the operator failed in the majority of mutants. Instead, a poor imitation developed containing only some of the recognition signals for the coat protein. Three mutants were of particular interest in that they contained double nonsense codons in the lysis reading frame that runs through the operator hairpin. The simultaneous reversion of two stop codons into sense codons has a very low probability of occurring. Therefore the phage solved the problem by deleting the nonsense signals and, in fact, the complete operator, except for the initiation codon of the replicase gene. Several revertants were isolated with activities ranging from 1% to 20% of wild type. The operator, long thought to be a critical regulator, now appears to be a dispensable element. In addition, the results indicate how RNA viruses can be forced to step back to an attenuated form.

INTRODUCTION

The single-stranded RNA coliphages have an incredible speed of reproduction. Starting from a single infectious particle, exponential amplification to 10^{12} to 10^{13} phages/ml can be obtained in a few hours. Combined with a low accuracy of replication (Domingo *et al.,* 1985) this implies a high evolutionary rate, and it can be assumed that the genome has reached the "state of comfort," meaning that no further improvements can be made. Every nucleotide in the local sequence space has been tested for optimal performance. This inevitable strive to perfection applies equally well to the amino acid sequence of the encoded proteins as to the RNA higher-order structures that contribute to the reproductive success.

So far, we have been mostly interested in the role played by RNA structure in the control of translation and replication. We have shown that base changes precluding the formation of such regulatory structures lead to a loss of fitness (Klovins *et al.,* 1997, 1998; Olsthoorn *et al.,* 1994). The titer of such mutants decreases by many orders of magnitude, but on passaging, suitable alternatives for the lost control mechanism evolve. The number of possibilities an RNA genome has to compensate for such debilitating mutations seem large and thus it is not possible to construct simplified viruses by base substitutions that inactivate such controls. Still, a better understanding of the essence of an RNA bacteriophage requires that we can distinguish between those elements in the genome that are essential and those that only help to improve reproduction. That is, can we distinguish the basal framework from the structural ornaments that only speed up the infectious cycle? As a preliminary definition, we propose that ornaments can be deleted but the hard core cannot.

In this report, we make a start in this direction. The focus is a simple hairpin structure, studied by many researchers, and containing the translational start signals of the replicase gene. It is indicated as operator in Fig. 1B. Synthesis of the replicase protein takes place as a short burst early in infection, and control of translation has two aspects. First, two long-distance interactions shown in Fig. 1B as MJ and VD prevent ribosome binding unless these two interactions are temporarily broken up by ribosomes translating the coat gene (Min Jou, 1972; Berkhout and van Duin, 1985; van Himbergen *et al.,* 1993; Licis *et al.,* 1998). This mechanism installs the coat replicase translational coupling, formerly called translational polarity. The operator hairpin by itself is too weak to interfere with ribosome binding (Berkhout, 1985).

The second control becomes operative when the coat protein concentration in the infected cell reaches a sufficiently high level to favor its binding to the operator. As a consequence, the replicase gene is

[1] To whom reprint requests should be addressed. Fax: (71) 5274340. E-mail: j.duin@chem.leidenuniv.nl.

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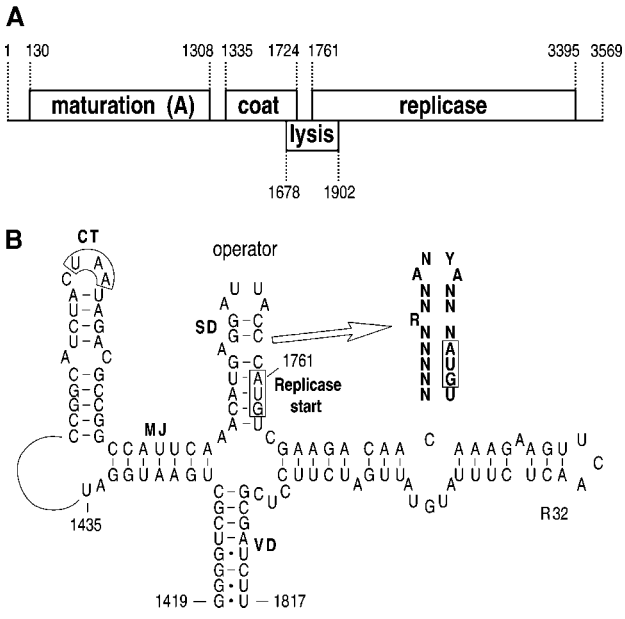

FIG. 1. (A) Genetic map of RNA phage MS2. (B) RNA secondary structure model around the start of the replicase gene. The structure is based on probing, phylogenetic comparison, and mutational analysis (Skripkin *et al.,* 1987; van Himbergen *et al.,* 1993; Licis *et al.,* 1998). The inset shows the mutations introduced in the operator hairpin. The consensus elements for coat protein binding are the letters in the loop as indicated and the bulged purine. The identity of the seven base-pairs is not important. CT indicates coat terminator hairpin; SD, Shine-Dalgarno sequence. MJ and VD are long-distance interactions that down-regulate translation of the replicase gene.

now permanently closed to any further translation. The interactions involved in this coat protein–RNA complex have been analyzed at atomic resolution (Valegård *et al.,* 1994).

The hairpin has still another role. The coat protein–RNA complex is believed to be the nucleation point of the encapsidation of the RNA (Pickett and Peabody, 1993). It was also shown that RNA that contained the operator hairpin was encapsidated at a lower coat protein concentration than RNA that did not have the operator (Witherell *et al.,* 1991).

Finally, it must be noted that this stretch of RNA is part of the reading frame of the lysis gene (Fig. 1A).

In view of its multifunctional role, this coat replicase intercistronic region seems an indispensable part of the MS2 genome. Here, we describe how the phage can be forced to delete this region to continue its existence as a permanently attenuated virus. Two stop codons in the lysis reading frame were introduced in the operator hairpin sequence. Apparently, the probability of producing a pseudorevertant in which the stop codons have changed to sense is too small to occur. Instead, a simple way to rescue the reading frame seemed to be the deletion of the segment with the nonsense codons. In fact, not only the stop codons but also the complete operator hairpin was removed, and this highly sophisticated structure is thus dispensable for the RNA coliphages. We discuss phage life without the operator.

RESULTS

The aim of these experiments was to partially randomize the operator hairpin of phage MS2 and to select and characterize viable revertants. The positions subject to change are shown in Fig. 1B. The system we applied *en route* to mutants and revertants was essentially as described earlier (Licis *et al.,* 1998). Briefly, we make use of an infectious cDNA copy of phage MS2 to introduce mutations of our choice. Plasmids carrying the wild-type MS2 cDNA generate spontaneously about 10^{11} PFU/ml on overnight growth in *Escherichia coli* F^- cells. Mutant cDNA produces less, depending on the changes. The mutations shown in Fig. 1B were introduced into an MS2 cDNA fragment, which was subsequently exchanged with its wild-type counterpart present in full-length infectious MS2 cDNA. The plasmid pool obtained *in vitro* was transformed to M5219 cells, which were seeded onto nitrocellulose filters placed on agar plates and grown overnight. Reinfection of M5219 hosts cannot take place because these bacteria lack F pili. Phage-producing colonies were identified by replica plating of the filters onto lawns of F^+ bacteria, where they usually yield halos. About 20% of the transformants produced phage. The size and clarity of the lysis halo correlated with the titer of the infectious mutant clone. We then visually selected 20 phage-producing colonies and sequenced the plasmid across the mutagenized region. The evolved revertants were sequenced at the beginning and at the apparent end of their evolution. Six instructive mutants are discussed here.

Repair of the operator hairpin

The first example represents the classic case. Mutant A16 contains three substitutions in the target region as shown by the gray squares in Fig. 2. These destroy the operator as well as the Shine-Dalgarno sequence, and

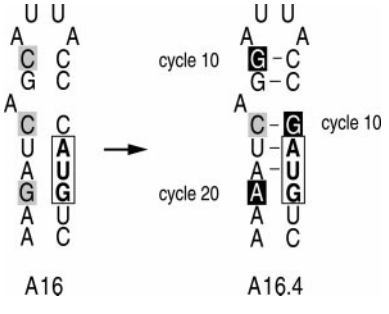

FIG. 2. Evolution of the operator hairpin in mutant A16. Introduced mutations are in gray squares. Adaptive mutations are in black squares.

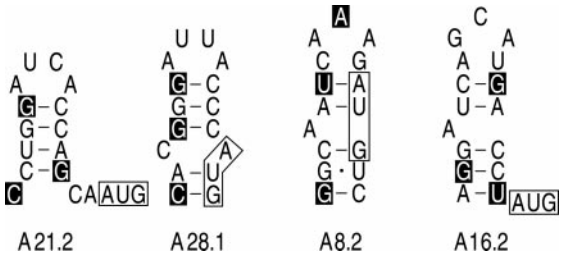

FIG. 3. Predicted structures at the replicase operator in four revertants after 20 cycles. The start codon is boxed, and the adaptive mutations are in black squares. The initial mutations are not shown but can be deduced from the wild-type sequence. The sequences shown represent the end of evolution. Note the variable position of the start codon in the putative operator. A16.2 derives from the same parent as A16.4 (Fig. 2).

thus, not surprisingly, the titer of this construct goes down. It is 10^8 PFU/ml compared with 10^{11} PFU/ml for the wild type. The sequence of the revertant (A16.4) shows three suppressor mutations (black squares). The C → G change near the loop is a reversion to wild type. It partly restores the SD sequence (AGGA), and it repairs the top of the hairpin. Then there is the C → G substitution at position −1 with respect to the start codon, which helps to rebuild the operator hairpin, although with a reversed base-pair. This result supports the studies showing that the identity of the base pairs is not important for coat protein binding (Witherell *et al.,* 1991; Schneider *et al.,* 1992). Finally, between cycles 10 and 20, we have a G → A change near the bottom of the stem. This substitution is not fully understood. Return to the wild-type base pair may not be possible because the weaker SD sequence in A16.4 needs a weaker hairpin stability (de Smit and van Duin, 1994; Olsthoorn *et al.,* 1995), but why the G must be changed to A is not clear. The operator hairpin of A16.4 is not a very good one. In the presence of coat protein, it represses replicase translation by a factor of 2, whereas reduction by the wild-type hairpin is ninefold under the same conditions. This malfunctioning is probably due to the remaining mismatch. Indeed, as reported by Witherell *et al.* (1991), shortening the stem lowers its affinity for the coat protein dimer.

Meanwhile, it should be noted that reconstruction of the operator hairpin was the exception rather than the rule. Most mutants were apparently unable to refind an operator homolog and had settled for the next best solution. In many cases, the hairpin structure that evolved carried the consensus loop sequence (ANYA) of four nucleotides but missed the bulged A. Alternatively, they did have a bulged A at the right position but missed the consensus loop. Instead, they contained a loop of five nucleotides (see Fig. 3 for four examples). These structures look very much like R32 (Fig. 1B). The suggestion is that these hairpins are still partly functional and have retained the capacity to bind to one monomer of the coat protein dimer.

Single nonsense codons in the lysis frame

AL7 and AL6 had 11 and 9 changes, respectively, in the operator (Fig. 4). In both mutants, the new sequence produced a nonsense codon in the lysis frame (underlined). Furthermore, the substitutions completely destroyed the operator hairpin as well as the SD sequence for the replicase. These mutants then have at least three or four problems. They do not make much replicase. They cannot shut off replicase synthesis. They lack the nucleation point for encapsidation, and they do not produce lysis protein. Evolution of these two mutants shows that the most serious problem is the lack of lysis protein. As shown in Fig. 4, this was the first thing to be repaired. Two plaques obtained from AL7 had the same G → U transversion that removed the stop codon. Sometimes the change from nonsense to sense could be combined with improving the SD sequence (AL6.1) where eventually a GAG codon replaces the UAG stop codon. At cycle

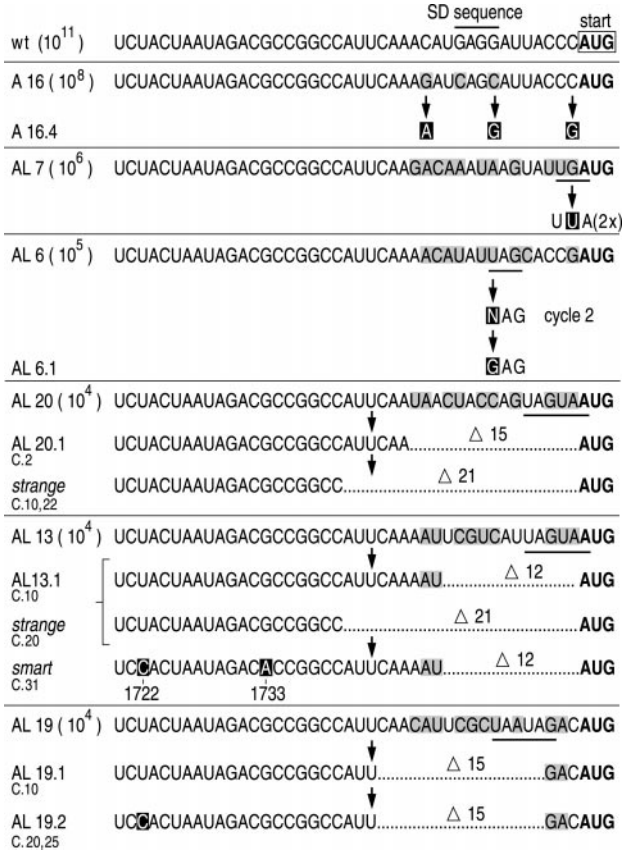

FIG. 4. Compilation of selected mutants and their evolution on passaging. In parentheses is the measured titer of the cDNA plasmid after overnight growth in F^- cells. Initial mutations are in gray. Adaptive substitutions are in black. Stop codons in the lysis frame are underlined. C.2 stands for cycle 2, and so on.

2, there still is a mixed sequence centered at the UAG stop codon. From this mixture, the GAG emerges as the winner, probably because this is the best SD sequence. Further evolution of these revertants was not carried out.

Double nonsense codons in the lysis frame

Three colonies produced very vague halos on the replica plates. When these cells were grown overnight in liquid medium, their supernatant contained only 10^4 PFU/ml. The plaques were small and developed with a delay of about 10 h compared with a few hours for the wild type. On sequencing the plasmids, we found that the random substitutions in the operator hairpin had caused two successive in frame nonsense codons in the three mutants (AL20, AL13, and AL19; Fig. 4). The titer of the single nonsense mutant AL6 is 10^5. This is a drop of 3 logs compared with mutants not having a stop codon, indicating that the probability to remove the stop is about 10^{-3}. The chance for two simultaneous nonsense-to-sense reversions will then be about 10^{-6}. Because the titer of all our hairpin mutants fluctuates around 10^8 PFU/ml (not shown), we expect a titer of 10^2 for the double stop mutants. The observed value of 10^4 PFU/ml therefore suggested a different solution to remove the stop codons. Indeed, the phage deletes part of its genome to restore the reading frame.

Let us first consider the simplest case, AL20. Its sequence in the structure model is shown in Fig. 5. The two stop codons are marked by a line. Sequencing of total phage RNA after the first infection (cycle 2) showed the presence of one kind of genome, the revertant AL20.1 (Figs. 4 and 5). AL20.1 had solved its lysis problem by deleting a 15-nt region that included both nonsense codons. After eight more passages, AL20.1 was taken over by a revertant called the *STRANGE*, which had lost an additional 6 nt. The *STRANGE* probably represents the end situation (i.e., we did not notice any further changes in the sequenced region in the next 12 cycles). With a deletion of 21 nt, the *STRANGE* is the shortest genome we encountered in the present study. This revertant has several apparent defects. It has lost the operator hairpin, it has no SD sequence in front of the replicase gene, it has a shortened version of the MJ long-distance interaction (Fig. 5), and it lacks seven amino acids at the N-terminal part of the lysis protein. This last defect is probably not very serious in that it was shown that the N-terminal 30 amino acids of the lysis protein are dispensable for the lytic function (Berkhout *et al.,* 1985). Indeed, when cloned back into the infectious cDNA copy, the *STRANGE* MS2 cDNA lysed the cells with only a slight delay compared with wild type on induction of the P_L promoter controlling transcription of the MS2 cDNA sequence (Fig. 6).

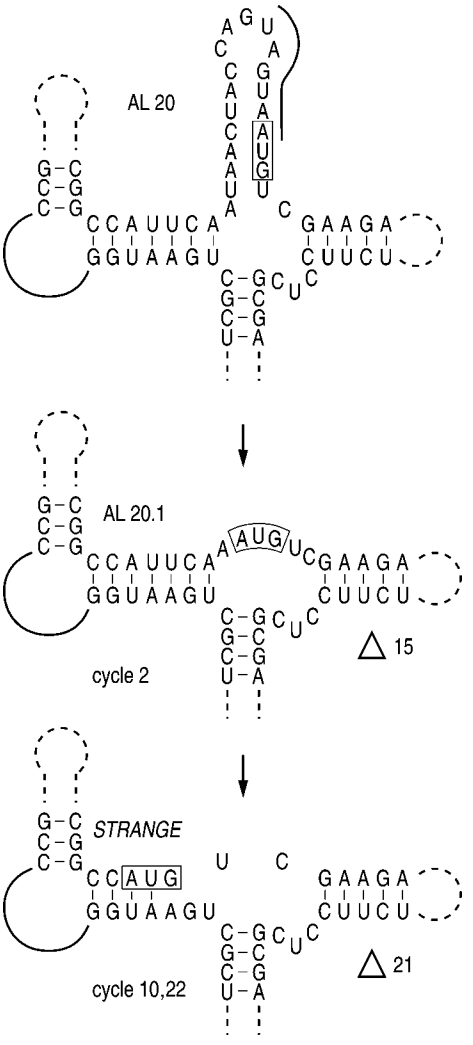

FIG. 5. Evolution of mutant AL20 shown in the context of the local RNA structure. The start codon is boxed, and the nonsense codons in the lysis reading frame are marked by a line.

AL13 (Fig. 4) has the same two stop codons as AL20 but other substitutions in the rest of the operator hairpin. At cycle 2, the progeny of this mutant consisted of a mixture of several unknown deletion variants as suggested by the ambiguous sequence at the relevant region. Nevertheless, AL13.1 carrying a 12-base deletion could be identified as the majority sequence, and it fully dominated the population at cycle 10. However, after 10 additional cycles, the *STRANGE* revertant transiently appeared, but five cycles later, this sequence became mixed with that of a revertant we call the *SMART*. After six more cycles, the *STRANGE* was outgrown by the *SMART*. The *SMART* is likely a descendant of AL13.1 that has accumulated two substitutions in the coat gene terminator hairpin (Figs. 4 and 8). Remarkably, the dominance of the *SMART* was accompanied by a shift from small plaques to medium-sized ones. There also

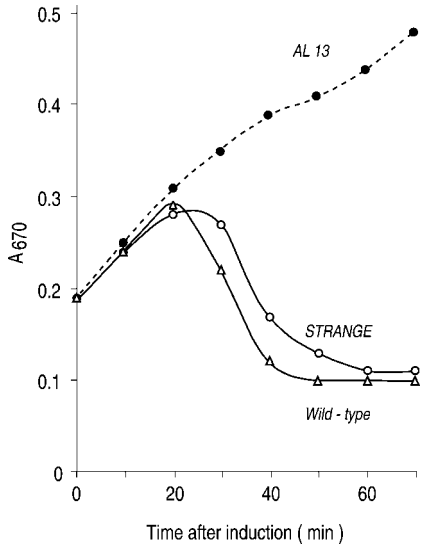

FIG. 6. Growth curves of *E. coli* cells after induction of the P_L promoter controlling transcription of the plasmid encoded MS2 cDNA. Curves show the efficiency of the lysis protein to lyse the host cell. AL13 has an early stop codon in the lysis reading frame. *STRANGE* has a seven-amino-acid deletion in the N-terminal region of the lysis protein.

was faster plaque development and an increase in phage titer to about 20% of wild type. It may be noted that *SMART* is unlikely to have evolved from *STRANGE* because this would require the insertion of an already lost sequence.

A confirmation of the fitness increase in the various revertants was obtained in an infection experiment (Fig. 7). *E. coli* F^+ cells were grown to early log phase, and the culture was split into five parts. One of these was left as a control, while the others were infected with AL13.1, *STRANGE*, *SMART*, and wild type at a phage/bacterium

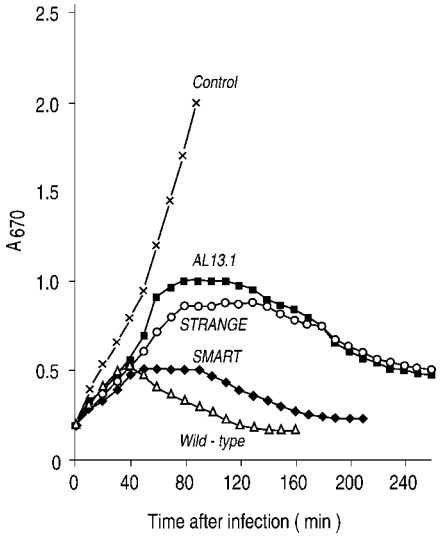

FIG. 7. Growth curves of F^+ *E. coli* cells infected with wild-type and mutant MS2 phages at a multiplicity of infection of 100. The curves show the time needed to lyse the cells.

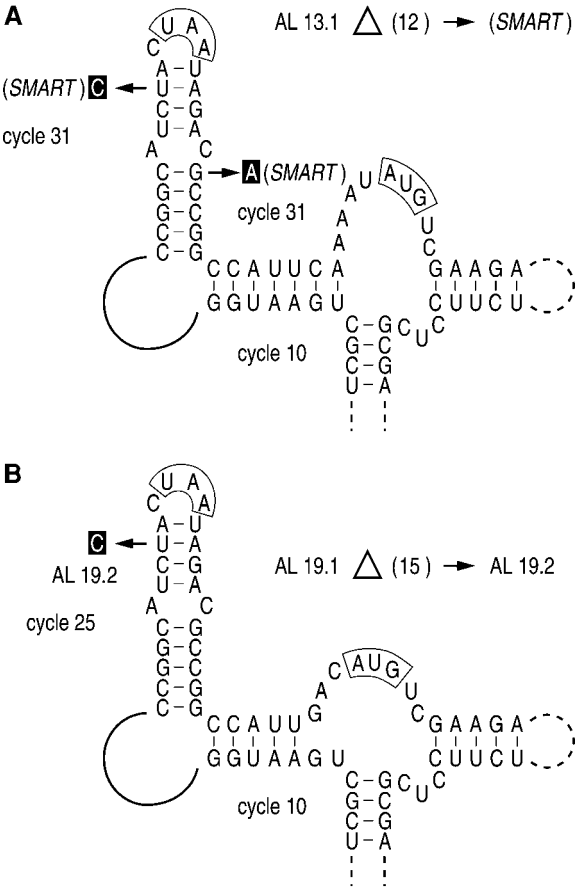

FIG. 8. Evolution of AL13 and AL19 shown here in the context of the local RNA structure. AL13 first deletes 12 nt (AL13.1) and subsequently adopts two mutations in the coat terminator hairpin (*SMART*). AL19 first deletes 15 nt (AL19.1) and then selects a mutation in the coat terminator hairpin (AL19.2).

ratio of 100. This excessive ratio was chosen to ensure that small inaccuracies in titer determination of the respective lysates would not influence the result. Of all cultures, A670 is then monitored with time. Cells infected with the wild-type MS2 lyse within 1 h. Infections with AL13.1 and the *STRANGE* phage are strongly retarded, whereas the *SMART* is only somewhat slower than wild type. As one would expect, the revertant that proliferates best is the one that is selected by evolution (i.e., the *SMART*).

Finally, we present the evolution of mutant AL19, which resembles that of AL13 (Fig. 4). The phage first made a 15-nt deletion to remove the stop codons and then selected a base change in the coat terminator hairpin (Fig. 8B). This mutation, U1722C, is the same as the one accumulated by the *SMART* revertant, and it substantially increased phage titer. Another similarity was that after the first infection (cycle 2), the bulk sequence contained many deletion variants (not shown).

TABLE 1

Expression of Replicase Measured as Miller Units in rep–lacZ Fusions

	MS2 cDNA preceding the *lacZ* gene	
Mutant	1365–2057	1628–2057
A16	1.0	57.4
A16.4	35.6	275.1
A13.1	1.1	11.8
SMART	44.4	382.3
STRANGE	1.3	n.d.

The suppressor mutations improve replicase translation

As pointed out above, our double nonsense mutants have three defects: no lysis protein, no operator hairpin, and a bad ribosome start site ahead of the replicase gene. We have seen that the first thing to be repaired is the lysis reading frame. The deletion to accomplish this need only be 6 nt but in fact includes virtually all of the operator hairpin, and its evolutionary reconstruction thus is clearly out of sight. For this reason, one would surmise that the adaptive mutations after the initial deletion are aimed at an increased replicase synthesis. We have analyzed this question for revertant AL13.1, which evolves to *SMART* by two substitutions in the coat terminator hairpin. Replicase synthesis was measured in replicase-lacZ protein fusions (see Materials and Methods). The cDNA constructs used started at either nt 1365 or 1628 and extended to the replicase-lacZ fusion point at nt 2057 of the MS2 map (Fig. 1A). The fragments were chosen not to contain the start of the coat gene, and we thus measured replicase synthesis potentially repressed by the MJ and VD long-distance interaction in clones starting at nt 1365. In clones starting at nt 1628, both of these interactions cannot form, and we measured the unrepressed intrinsic activity of the replicase ribosome binding site. As seen in Table 1, *SMART* has a substantially increased replicase production compared with AL13.1. This is indeed what would be expected of these secondary mutations. That replicase synthesis in the clones starting at nt 1365 is lower than in clones beginning at nt 1628 shows that the translational coupling in AL13.1 and *SMART* must still be intact. The deletion of the operator apparently leaves the MJ and the VD base-pairing undisturbed.

As a reference, we also measured the revertant AL16.4. Its capacity to produce replicase is about the same as that of the *SMART* revertant. In contrast, the *STRANGE* revertant produces almost no replicase even though we evolved it for about 25 cycles.

DISCUSSION

In this study, we randomly mutagenized the operator hairpin ahead of the replicase gene of MS2 RNA. This structure element can bind a coat protein dimer, which then shuts off further initiation of translation. The protein–RNA complex is also considered the nucleation point for encapsidation of the RNA. Three of our mutants contained double stop codons in the lysis gene reading frame of which the operator hairpin is a part.

All three mutants solved the nonsense codon problem by a variable deletion that removed the stop signals. Two important results emerge from the study. First, the operator hairpin is not essential. Second, a virus can be forced to delete portions of its genome by the introduction of double stop codons. This can be a valuable tool to prepare permanently attenuated strains. Construction of attenuated strains by multiple substitutions may fail because the return to wild type cannot be excluded while the pathway may not be irreversibly blocked. In contrast, deletions are difficult to repair. As a first step, this requires duplication of a nearby region to fill in the gap (Olsthoorn and van Duin, 1996a), but if the new sequence is constrained while it must form a precise structure to be useful, the chances of returning to wild type become so small that such revertants will in all probability not be present in the progeny pool.

Deletions in the genome of phage MS2 are not unprecedented. Olsthoorn and van Duin (1996a) constructed a mutant that had a 19-nt deletion in the intercistronic region between maturation and coat protein. Its viability was about 10 logs lower than wild type. One of the revertants had deleted an additional six nucleotides, which led to a better SD sequence for the coat gene.

In the present study, all three double nonsense mutants have the stop codons in tandem and a deletion of six nucleotides would suffice. However, the deletions we find are always larger, suggesting that the minimal deletions are not viable.

The operator hairpin is dispensable

The interaction between the MS2 coat protein and the operator is probably the best studied and defined protein–RNA complex (Witherell *et al.,* 1991; Valegård *et al.,* 1994). This circumstance has somehow been conducive to the idea that this regulatory design must be essential. It is thus unexpected that the phage simply deletes the hairpin at only a small cost (*STRANGE* and *SMART* phages had titers of 2% and 20% of wild type). This result suggests that many sophisticated controls have developed only while they add enough to fitness to outgrow competitors, but in the absence of competition, their contribution to proliferation may be small.

In fact, the dispensability of the operator hairpin is not a complete surprise. Peabody (1997) came to a similar

conclusion when he evolved an MS2 mutant of which the coat protein had superrepressor activity (i.e., it bound stronger and thus in an earlier phase of infection to the operator hairpin). As a consequence, replicase synthesis was expected to be prematurely shut off. Most of the revertants contained suppressor mutations in the operator hairpin that reduced or possibly destroyed its ability to bind coat protein. In these revertants, a mutant form of the operator hairpin was still present, and weak coat protein binding could not be rigorously excluded. Now, our deletion mutants take away the last shred of doubt: the phage can live without the operator. Packaging of the RNA in such operatorless revertants was proposed to occur via secondary coat protein-binding sites (Peabody, 1997).

Understanding the suppressor mutations

It is difficult is to interpret the size of the deletions. If only six nucleotides will restore the lysis frame, why is additional material, up to 21 nt, removed? Apart from the inevitable contribution of chance to the size of such random deletions, we propose that another factor plays a role. RNA phages were shown to be negatively affected by stretches of unstructured RNA, and such sequences were quickly removed from the genome (Olsthoorn and van Duin, 1996b). Thus the removal of only six nucleotides might leave a large unstructured loop in place of the operator hairpin. In a previous study, we deliberately removed half of the maturation gene terminator hairpin and half of the coat protein initiator hairpin (Olsthoorn and van Duin, 1996a). The evolutionary response was either a duplication of a bordering sequence to reconstruct both stem-loops or a further deletion to sacrifice the terminator and rebuild the initiator with the remaining nucleotides.

Another question is why AL20.1 would delete an additional 6 nt to become the *STRANGE*, whereas AL19.1, which is identical to AL20.1 except for 2 nt, evolves a base change in the coat terminator hairpin. We have no reasonable answer, and we suppose that the different pathways are simply the result of chance (i.e., AL19.1 could also have evolved in the direction that AL20.1 chose, and vice versa). At any rate, both AL19.1 and AL20.1 are under pressure to augment their replicase synthesis. To understand how they accomplish this, we must realize that the initial deletion of 15 nt has made the coat terminator hairpin a part of the ribosome binding site of the replicase gene. The thermodynamic stability of the terminator hairpin is henceforth an important factor in replicase translation. With this perspective, the U1722C substitution is expected to boost replicase synthesis. The same strategy is chosen by AL13.1. Here, we find two destabilizations to upgrade the replicase start resulting in the *SMART* revertant. It is intriguing that the *SMART* revertant also has selected U1722C, a mutation that not only destabilizes the terminator hairpin but also gives rise to a different C-terminal amino acid in the coat protein. Quite possibly the rationale for this mutation transcends our present understanding of the RNA phages.

The *STRANGE* revertant seems to have found a radically different solution. We suppose that the additional 6-nt deletion couples replicase translation to termination of coat gene translation. The new distance between stop and start is only 12 nt. Here, we may recall that lysis expression is also coupled to termination at the coat stop (Berkhout *et al.,* 1987; Adhin and van Duin, 1990).

We should realize that what all these revertants have lost is the nucleation site for capsid formation and the capacity to completely shut off replicase. The control over replicase synthesis by coat gene reading has been maintained (Table 1). Although in wild type there is a short burst of replicase synthesis, we expect a slow accumulation of the enzyme in the revertants that parallels the build-up of the coat protein supply. In addition, in the absence of the operator, one would expect packaging to take place at a later phase in infection, with the nucleation site now taken over by another, so far unidentified structure or structures.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmids were grown in *E. coli* K12 strain M5219, encoding the thermosensitive λ repressor (cI857) and the transcriptional antitermination factor N (Remaut *et al.,* 1981). Evolution was performed in *E. coli* F^+ AB259 (Hfr3000, thi^-, su^-) cells. Bacteria were grown on LB broth.

Infectious MS2 cDNA clones contain a complete copy of the phage genome downstream of the thermoinducible P_L promoter of phage λ. M5219 bacteria bearing the infectious cDNA produce the phage even in the absence of induction. Wild-type construct pMS2000 yields about 10^{11} PFU/ml after overnight growth in liquid broth (Olsthoorn *et al.,* 1994). Plasmid pD is a derivative of pMS2000 that carries a short linker in place of the *Xba*I (1303)–*Bfr*I (1901) fragment (Licis *et al.,* 1998). This plasmid does not produce viable phage and was used to reconstruct the infectious clone by the exchange with any mutated 1303–1901 phage genome fragment. Clones used for the measurements of replicase gene expression contained the specified MS2 cDNA fragments fused to the *lac*Z gene at the *Bam*HI site (position 2057 of the phage cDNA) behind the P_L promoter (van Himbergen *et al.,* 1993).

Mutagenesis

Mutations in the 1746–1760 region of the MS2 genome were introduced with oligonucleotide RP57 (GAACT-

TCTTTGTTGTCTTCGACATNNNTRNTNNYNNNNNTTGA-ATGGCCGGCGTCTA, where N stands for any nucleotide, R is purine, and Y is pyrimidine), which corresponds to the 1728–1784 MS2 cDNA sequence. At first, the flanking regions 1200–1745 and 1765–2067 MS2 cDNA were amplified using primer p180 (identical to the 1200–1217 region of the MS2 genome), p162 (complementary to 1728–1745), p9 (identical to the 1765–1784 MS2 sequence), and p114 (complementary to 2047–2067). These fragments were gel-purified, precipitated, and dissolved in 50 μl of water. In the mutagenesis step, the reaction mixture (40 μl) was combined from 5 μl 10× PCR buffer (100 mM Tris, pH 8.8, 500 mM KCl, 0.8% Nonidet P-40), 4 μl of 25 mM $MgCl_2$, 1 μl of BSA (1 mg/ml), 8 μl of 1 mM dNTPs, 5 μl of 5 μM primer p180, 5 μl of the 1765–2067 MS2 cDNA fragment, 2 μl of 0.5 μM oligonucleotide RP57, 2 U of *Taq* DNA polymerase, and water. After 5 cycles (denaturation at 95°C for 30 s, annealing at 45°C for 40 s, and elongation at 72°C for 40 s), 5 μl of the 1200–1745 MS2 cDNA fragment and 5 μl of 5 μM primer p114 were added, and the reaction was continued for an additional 25 cycles. The resulting PCR product was precipitated, dissolved in water, and after the treatment with *Xba*I and *Bfr*I restriction endonucleases, ligated to the pD plasmid cleaved with those same enzymes. DNA was electroporated into *E. coli* M5219 bacteria.

Selection of the infectious clones and recording phage evolution

The infectious clones were selected and phage evolution was recorded essentially as described earlier (Licis *et al.,* 1998). Transformed *E. coli* M5219 cells were seeded onto nitrocellulose filters placed on top of LB agar plates. After overnight growth of bacteria, the phage-producing colonies were identified by replica plating of the nitrocellulose filters on lawns of AB259 F^+ cells. A selected number of infectious clones were picked and grown in liquid broth at 28°C. The cells were used to isolate plasmids for sequence analysis using primer p108 (identical to the 1628–1648 MS2 sequence), whereas the supernatant was the source of the phage, which was used to infect F^+ bacteria. The creation of phage from the plasmid in the F^- host is defined as cycle 1, the first infection in F^+ cells is defined as cycle 2, and so on. Typically, evolution was allowed to proceed for 10–30 cycles. About 10^3 to 10^5 PFU of the phage was used for the first infection of F^+ AB259 bacteria and 10^5 PFU was used for each subsequent infection. Phage RNA was sequenced after various cycles to record the evolutionary adaptations by suppressor base changes. The sequence analysis was carried out after RT-PCR amplification of the 1200–2067 MS2 cDNA fragment, routinely using primer p108. In some cases, primer p105 (complementary to the 1572–1585 MS2 sequence) was used to check the coat gene part of the MJ interaction.

Detection of lysis and replicase gene expression

E. coli M5219 cells that harbor MS2 cDNA behind the P_L promoter were grown at 28°C until an A_{670} of about 0.25 was reached. Then the P_L promoter was induced by shifting the cultures to 42°C. To monitor lysis gene expression, the cell density was recorded at A_{670}. To measure replicase expression, cultures were placed on ice after 30-min induction for at least 10 min, and the β-galactosidase activity in 0.4-ml samples was determined according to standard procedures (Miller, 1972) except that the cell densities were measured at A_{570} and the turnover of ONPG at A_{670}. All measurements were performed on microtiter plates.

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